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B cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) mediate CD40-independent help by memory CD4 T cells

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Abstract

Donor-reactive memory T cells undermine organ transplant survival and are poorly controlled by immunosuppression or costimulatory blockade. Memory CD4 T cells provide CD40-independent help for the generation of donor-reactive effector CD8 T cells and alloantibody that rapidly mediate allograft rejection. The goal of this study was to investigate the role of B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) in alloresponses driven by memory CD4 T cells. The short-term neutralization of BAFF alone or BAFF plus APRIL synergized with anti-CD154 mAb to prolong heart allograft survival in recipients containing donor-reactive memory CD4 T cells. The prolongation was associated with reduction in anti-donor alloantibody responses and with inhibited re-activation and helper functions of memory CD4 T cells. Additional depletion of CD8 T cells did not enhance the prolonged allograft survival suggesting that donor-reactive alloantibodies mediate late graft rejection in these recipients. This is the first report that targeting the BAFF cytokine network inhibits both humoral and cellular immune responses induced by memory CD4 T cells. Our results suggest that reagents neutralizing BAFF and APRIL may be used to enhance the efficacy of CD40/CD154 costimulatory blockade and improve allograft survival in T cell-sensitized recipients.

Introduction

The presence of donor-reactive memory T cells prior to transplantation results in robust immune responses to transplanted organs and poor allograft outcome (1, 2). Compared to naïve T cells, memory T cells are less susceptible to currently used immunosuppression or costimulatory blockade approaches. We have previously reported that donor-specific memory CD4 T cells contribute to allograft rejection by providing help for activation of naïve donor-reactive CD8 T cells and for alloantibody (alloAb) production that, in turn, mediate allograft injury and rejection (3–5).

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Supplemental Information

Additional Supporting Information may be found in the online version of this article.

During primary immune responses, helper functions of CD4 T cells are critically dependent on CD154/CD40 interactions. In contrast, memory CD4 T cells provide help to CD8 T cells and to B cells and induce allograft rejection in a CD40-independent manner (3, 4, 6). While several agents targeting the CD40/CD154 pathway are currently being developed, our previous findings raised concerns that these approaches will fail to inhibit pathogenic helper functions of memory CD4 T cells and should be accompanied by strategies controlling CD40-independent anti-donor immune responses in T cell-sensitized patients.

The challenge of inhibiting memory CD4 T cells necessitates the development of therapies targeting both memory CD4 T cells and the cells requiring their help. The TNF family members BAFF (B cell activating factor belonging to the TNF family) and a proliferation inducing ligand (APRIL) play critical roles in modulating lymphocyte survival, activation, and differentiation. These cytokines are produced by multiple cell types including stromal cells within secondary lymphoid organs, monocytes, macrophages, dendritic cells, and activated T cells, but not by cells of the B cell lineage (7). Ligand-receptor interactions within the BAFF cytokine network are redundant, with BAFF binding to BAFFR, TACI and BCMA, and APRIL interacting with TACI, BCMA and proteoglycans. All of these receptors are expressed by B cells at various stages of B cell development (8, 9). In addition, BAFF-R is expressed on activated and memory T lymphocytes and provides costimulatory signals to T cells (7, 10–13).

The best studied functions of the BAFF/APRIL cytokine network relate to B cell homeostasis and function. BAFF and/or APRIL neutralization decreases B cell numbers, prevents B cell activation, reduces Ab production and ameliorates disease in multiple animal models of autoimmunity (9, 14–16). In clinical transplantation, elevated serum levels of BAFF are a risk factor for renal allograft dysfunction, the development of anti-donor alloAb, and Ab-mediated rejection (17, 18). In a mouse model of islet transplantation, BAFF-neutralizing mAb combined with low dose rapamycin induced long-term allograft survival associated with decreased alloAb production and CD4 T cell activation (19). BAFF-deficient recipients treated with cyclosporin A had prolonged heart allograft survival compared to treated wild type mice (13). However, the therapeutic potential of BAFF/APRIL targeting reagents in sensitized recipients with pre-existing donor-specific memory T cells has not been previously tested.

The goal of this study was to investigate the role of BAFF and APRIL in helper functions of donor-reactive memory CD4 T cells. Due to the complexity of donor-specific responses in sensitized recipients and to the broad spectrum of BAFF/APRIL effects on various B cell populations, we used an adoptive transfer approach to focus on memory T cells in the context of naïve B cell repertoire. We report that short-term neutralization of either BAFF alone or BAFF plus APRIL synergizes with anti-CD154 mAb treatment to markedly prolong heart allograft survival in recipients containing donor-reactive memory CD4 T cells that are resistant to the effects of anti-CD154 monotherapy. This prolongation is associated with decreased memory CD4 T cell helper functions as evidenced by decreased *de novo* B cell and T cell responses to donor antigens. This is the first evidence that targeting BAFF family cytokines inhibits both humoral and cellular immune responses and significantly

delays costimulatory blockade resistant rejection in recipients containing donor-reactive memory T cells.

Materials and Methods

Animals

Female C57Bl/6 (B6, H-2^b), B6.SJL-*Ptprca*^a *Pep3^b*/BoyJ (B6.CD45.1) and male BALB/cJ (BALB/c, H-2^d) and SJL/J-*Pde6b^{rd1}* (SJL, H2^s) mice, aged 6–8 weeks, were purchased from The Jackson Laboratory (Bar Harbor, ME). C57Bl/10NA;-(Tg) TCR Marilyn-(KO) Rag2 N11, N2 mice (Mar, H-2^b) were provided by Dr. Polly Matzinger (NIH) and crossed onto B6.CD45.1 background. All animal procedures were approved by the Institutional Animal Care and Use Committee at Cleveland Clinic.

Generation of alloreactive memory CD4 T cells

Memory Mar CD4 T cells were generated as previously published (3). Briefly, spleen cells from young (4–6 weeks) Mar female mice were stimulated in vitro with 3 μ M HY_{Dby} peptide (NAGFNSNRANSSRSS, Research Genetics, Huntsville, AL). After 4 days, the cells were washed, counted and intravenously injected into naïve B6 female mice (5 $\times$ 10⁶ cells/mouse). Animals were rested for 3 weeks prior to heart transplantation.

Cardiac transplantation and recipient treatment

Vascularized heterotopic cardiac allografts were transplanted and monitored as previously described (3, 4). Rejection was defined as a loss of palpable heartbeat and confirmed by laparotomy. Grafts were harvested at the time of rejection, embedded in paraffin and stained with H&E, anti-CD3 and anti-C4d Ab. Anti-CD154 Ab MR1 (Bio X Cell, West Lebanon, NH) was administered on day -1 (1 mg i.v.). Mouse Fc fusion proteins mBAFFR-mFc4, mTACI-mFc4 and an Fc-matched control (mFc4), all containing Fc mutations that eliminate ADCC and CDC effector functions (ZymoGenetics, Seattle, WA), were injected on days 2, 4, 6, 9, 11, and 13 post-transplant (0.1 mg i.p.). Recipient CD8T cells were depleted using a cocktail of monoclonal anti-mouse CD8 Ab (clones YTS169 and TIB105, Bio X Cell) with 0.2 mg of each Ab injected i.p. on days -3, -2, -1 before transplantation and then every 5 days throughout the duration of the experiment. The efficiency of CD8 T cell depletion (>98% compared to non-treated mice) was confirmed by flow cytometry at the time of sacrifice.

Flow cytometry

Fluorochrome-conjugated Ab were purchased from BD Pharmingen (San Diego, CA) or from eBioscience (San Diego, CA). Cells were isolated from peripheral blood or spleen and stained as previously described (5, 6). At least 50,000 events/sample were acquired on a BD Bioscience FACSC alibur (BD Biosciences, San Diego, CA) followed by data analysis using FlowJo software (Tree Star Inc., Ashland, OR).

Measurement of alloantibody titers

Donor- and third party- reactive antibody titers in recipient serum were determined on day 14 post-transplant and at the time of graft rejection as previously published (20). Briefly, donor BALB/c and third party SJL thymocytes were incubated with 100µl of serially diluted serum. Fluorochrome-conjugated goat anti-mouse IgG2b, anti-mouse IgG1, anti-mouse IgG2c, and anti-mouse IgG3 were used as detecting antibodies at a 1:50 – 1:100 dilution (all from BD Pharmingen). Cells were washed and analyzed by flow cytometry. The titer of BALB/c-reactive IgG alloantibody in serum was calculated as previously published (5, 21). For each IgG isotype, the dilution that returned the mean fluorescence intensity (MFI) to the level observed with a 1:90 dilution of naïve B6 serum was divided by two and reported as the titer.

ELISPOT assays

IFN γ , IL-4, and IL-17 ELISPOT assays were performed as previously described using capture and detecting anti-mouse cytokine antibody from BD Pharmingen (3–5, 22). Mouse IL-21 ELISPOT assay kit was purchased from eBioscience and used per manufacturer's instructions. Recipient spleen cells were stimulated with mitomycin C-treated BALB/c or SJL spleen cells or with 3µM HY_{Dby} peptide for 24 h. The frequencies of IgG and IgM secreting ASCs were determined using ELISpot^{PLUS} for mouse IgG or IgM kits (MABTECH AB, Nacka Strand, Sweden) as previously published (20). The resulting spots were analyzed using an ImmunoSpot Series 4 analyzer (Cellular Technology, Cleveland, OH).

Statistical analysis

Heart allograft survival was compared between groups by Kaplan-Meier analysis. Differences between groups during recall immune responses were analyzed using a non-parametric equivalent of one-way ANOVA, the Kruskal-Wallis test. When the overall p value was <0.05, pairwise comparisons were carried out using Mann-Whitney test. A value of p<0.05 was considered statistically significant.

Results

Neutralization of BAFF or BAFF/APRIL synergizes with anti-CD154 mAb to prolong heart allograft survival in CD4 T cell sensitized recipients

B6 recipients containing donor-reactive memory CD4 T cells were generated as we previously published (3, 4, 6). TCR transgenic Mar T cells specifically respond to HY_{Dby} peptide presented by I-A^b but not to H-2^d BALB/c alloantigens. After placement of male allogeneic heart transplants into C57BL/6 (B6, H-2^b) female recipients, transferred memory Mar T cells recognize the donor HY_{Dby} peptide presented by female antigen presenting cells through the indirect pathway. Upon reactivation, memory Mar cells provide help for naïve CD8 T cell activation and the production of donor MHC-reactive alloAb similarly to polyclonal memory CD4 T cells (3).

Recipients containing memory Mar cells received BALB/c male heart allografts and were treated with anti-CD154 mAb MR1. To test the roles of BAFF and APRIL, MR-1 treated

recipients were injected with soluble mouse Fc fusion proteins that neutralize either BAFF alone (mBAFFR-Fc) or both BAFF and APRIL (mTACI-Fc) for two weeks after transplantation (23). Consistent with our previous findings, treatment with MR1 alone or in combination with control fusion protein mFc4 failed to induce long term graft survival (MST of 17 and 11 days, respectively; Figure 1). In contrast, MR1 plus mBAFFR-Fc significantly prolonged heart allograft survival despite the presence of donor-reactive memory CD4 T cells (MST of 51 d.). Simultaneous neutralization of BAFF and APRIL with mTACI-Fc combined with MR1 was even more efficient in inhibiting allograft rejection (MST of 57 d., with 3 of 8 recipients maintaining viable allografts >90 d.) Notably, recipients containing memory CD4 T cells and not treated with MR1 uniformly rejected heart allografts regardless of BAFF/APRIL neutralization indicating that mBAFFR-Fc and TACI-Fc mainly target CD40-independent memory helper T cell functions (Figure 1B).

Neutralization of BAFF or BAFF/APRIL inhibits CD40-independent alloAb induction by memory CD4 T cells

We previously established that donor-reactive memory CD4 T cells mediate costimulatory blockade-resistant heart allograft rejection through at least two distinct mechanisms – generation of alloAb and activation of alloreactive CD8 T cells. We evaluated how neutralizing BAFF and APRIL affected B cell responses induced by memory CD4 T cells. Treatment with either mBAFFR-Fc or mTACI-Fc reduced the percentages and absolute numbers of B220⁺ cells in peripheral blood and spleen by d. 14 post-transplant compared to non-transplanted mice and control treated recipients (Figure 2B, Figure S1 and not shown). In the spleen, this reduction was mostly due to the decrease in B220⁺CD21/35⁺IgM⁺ follicular and B220⁺CD21/35^{hi}IgM^{hi} marginal zone B cells, but not in B220⁺CD21/35^{lo}IgM⁺ transitional and B220⁺CD38⁻GL7⁺ germinal center B cells (Figure 2). Recipients treated with mTACI-Fc had lower numbers of B220⁺CD138⁺ plasma cells in the spleen than mBAFFR-Fc treated or untreated groups (Figure 2). Treatment with mTACI-Fc, but not with mBAFFR-Fc, led to further decline in peripheral B cell numbers by d. 21 (Figure S1).

Consistent with our previous studies (3, 5, 6), donor-reactive memory T cells induced high serum titers of anti-donor IgG2c, IgG2b, IgG1 and IgG 3 alloAb despite treatment with MR1 Ab (Figure 3, open circles). Compared to control groups, mBAFFR-Fc slightly decreased serum titers of all analyzed IgG isotypes at d. 14 post-transplant, but differences were statistically significant only for IgG2c and IgG2b Ab (Figure 3). In contrast, simultaneous neutralization of BAFF and APRIL completely abrogated early generation of donor-reactive IgG as minimal serum Ab titers were detected on days 10 and 14 post-transplant (Figure 3 and data not shown). The reduced serum titers of anti-donor IgG alloAb at d. 14 posttransplant correlated with the ability of BAFF or BAFF/APRIL neutralization to extend graft survival in these recipients. Despite differences in early alloAb generation, mBAFFR-Fc and mTACI-Fc-treated recipients had comparable titers of donor-reactive IgG at the time of graft rejection. The serum levels of IgG2c, IgG2b and IgG3 were significantly decreased in both mBAFFR-Fc and mTACI-Fc treatment groups compared to mFc4 treated or untreated controls. However, recipients treated with either mBAFFR-Fc or mTACI-Fc developed high titers of anti-donor IgG1 Ab similar to those in control groups.

BAFF or BAFF/APRIL neutralization inhibits anti-donor T cell responses in recipients containing memory CD4 T cells

To evaluate phenotype and functions of memory helper T cells, we adoptively transferred CD45.1 congenic Mar memory cells into CD45.2 B6 recipients. Neither mBAFFR-Fc nor mTACI-Fc impaired long-term cell survival of transferred memory CD4 T cells (Figure S2A). Analysis at day 14 posttransplant showed that transferred Mar cells in all groups were uniformly CD44^{hi}CD25^{lo} (Figure 4A). About 50% Mar cells in control MR1-untreated recipients expressed CD69, a marker of recent antigen activation. Treatment with MR1 + mTACI-Fc significantly decreased the numbers of CD69^{hi} Mar cells (15±6%) while MR1 or MR1+mBAFFR-Fc treatment did not alter CD69 expression (46±9% and 52±4% of Mar cells, respectively, Figure 4A).

To measure priming of endogenous alloreactive T cells and re-activation of transferred memory Mar cells, we tested the ability of recipient splenocytes to produce effector cytokines following stimulation with donor BALB/c cells or HY_{Dby} peptide, respectively. As in our previous studies, adoptively transferred Mar memory T cells induced strong anti-donor IFN γ production by endogenous T cells despite treatment with anti-CD154 mAb (even though transferred Mar cells do not recognize BALB/c alloantigens directly). Additional treatment with mTACI-Fc not only inhibited generation of donor-reactive effector T cells but also reduced IFN γ production by preexisting memory CD4 T cells compared to untreated or mFc4 treated controls (Figure 5). mBAFFR-Fc administration had modest effects on early T cell responses, but markedly reduced frequencies of anti-donor and anti-HY_{Dby} IFN γ secreting T cells at the time of graft rejection. The frequencies of endogenous donor-reactive and memory Mar cells secreting IL-4 or IL-17 were markedly lower than those of IFN γ producers and were not significantly influenced by BAFF/APRIL neutralization at d. 14 or at rejection (Figure S2B and not shown).

We next analyzed subsets of ICOS⁺PD-1⁺ follicular helper cells within transferred Mar and endogenous CD4 T cell populations. MR1 treatment decreased numbers of endogenous but not memory Mar cells with Tfh phenotype. mTACI-Fc combined with MR1 reduced the numbers of Tfh cells within both endogenous and transferred memory subsets compared to MR1 monotherapy, while the addition of mBAFFR-Fc to MR1 treatment had no impact on Tfh numbers (Figure 5A). Furthermore, neutralizing BAFF/APRIL reduced the numbers of cells producing Tfh signature cytokine IL-21 in response to donor stimulator cells or HY_{Dby} peptide (Figure 5B).

Delayed heart allograft rejection after BAFF or BAFF/APRIL neutralization is mediated by donor-reactive alloantibody

Histological examination of heart allograft tissue revealed that the early allograft rejection in control recipients containing memory CD4 T cells and treated with MR1 was characterized by heavy mononuclear cell infiltration with many CD3⁺ cells and minimal C4d deposition on intramyocardial capillaries (Figure 6). In contrast, heart allografts prolonged by mBAFFR-Fc or mTACI-Fc plus MR1 treatment had low to moderate CD3⁺ cell infiltration consistent with the decreased anti-donor T cell responses in these recipients. However, these grafts had diffuse capillary C4d deposition and were heavily infiltrated by galectin-3⁺ cells

(Figure 6 and not shown) suggesting Ab-mediated injury as a prominent component of rejection. To assess the contribution of effector T cells versus pathogenic anti-donor alloAb during delayed rejection, we performed analogous experiments in recipients depleted of CD8 T cells prior to transplantation. Additional depletion of CD8 T cells had minimal further effect on graft survival (Figure 7 vs Figure 1) implicating donor-reactive IgG1 alloAb as the primary mechanism of late graft rejection in these recipients.

BAFF/APRIL neutralization inhibits *de novo* B cell responses but fails to prolong heart allograft survival in recipients containing preexisting alloAb

We next tested the efficacy of BAFF/APRIL neutralization in B6 recipients previously sensitized by BALB/c skin transplantation to elicit both cellular and humoral anti-donor immune responses. As heart allograft rejection in such recipients is promptly mediated by reactivated memory CD8 T cells (24), we additionally treated them with depleting anti-CD8 mAb. Consistent with our previous findings, anti-CD154 Ab treatment failed to induce long-term heart allograft survival in these recipients even despite CD8 T cell depletion (MST of 5.5 d., n=7). Neutralizing BAFF or BAFF/APRIL had no significant effect on the kinetics of rejection (MST of 6 d. with n=5 for both groups).

Skin-sensitized recipients had high serum titers of donor-reactive IgG alloAb prior to heart transplantation that were not altered by MR1 monotherapy (Figure 8A). Notably, analogous to the effects of BAFF/APRIL neutralization in recipients injected with donor-reactive memory CD4 T cells, addition of mTACI-Fc to MR1 treatment inhibited *de novo* generation of antibody secreting cells reactive to donor MHC class I (D^d) and class II (I-A^d) molecules (Figure 8B). However, this was not sufficient to significantly lower the serum titers (Figure 8A) and prevent the pathogenic function of pre-existing donor-reactive alloAb as evidenced by diffuse C4d deposition in graft capillaries at the time of rejection (Figure 8C).

Discussion

We previously reported that costimulatory blockade-resistant cardiac allograft rejection by memory CD4 T cells occurs through two major effector mechanisms – generation of pathogenic donor-reactive alloAb and newly activated effector CD8 T cells (3–6). We reasoned that limiting B cell survival and activation factors under these conditions would interfere with alloAb generation and extend allograft survival. Indeed, BAFF and APRIL appeared to be critical for the generation of long lasting, CD40-independent humoral immune responses. As anticipated, BAFF and APRIL neutralization decreased the overall numbers of B220⁺ cells in the periphery (the kinetics data indicate significant decrease between 10 and 14 days posttransplant, Figure S1). Consistent with previous reports, the decrease was most pronounced in follicular and marginal zone B cells but did not significantly affect transitional or germinal center B cells at this time (16, 25, 26). Notably, mBAFFR-Fc and mTACI-Fc had distinct effects on the kinetics of alloAb production. Administration of TACI-Fc inhibited the generation of donor-reactive IgG alloAb of all isotypes at early stages of the response. This can hardly be attributed to general B cell depletion as these recipients had significantly lower serum titers of anti-donor IgG alloAb compared to the control groups on day 10 post-transplant, when the effects of treatment on

B cell survival were not yet apparent (Figures 3 and S1). In contrast, neutralizing BAFF alone resulted in less prominent inhibition of IgG2c and IgG2b and had no significant effect on IgG1 and IgG3 titers at day 14 post-transplant. However, recipients treated with mBAFFR-Fc had decreased levels of IgG2c and IgG2b at the time of rejection.

The decrease in anti-donor alloAb responses can be attributed to several possible effects of BAFF or BAFF/APRIL neutralization. In addition to generation and homeostasis of the mature B cell compartment, BAFF and APRIL activate immunoglobulin isotype switching in mouse and human B cells even in the absence of T cells or CD40 signaling (27–29). Thus it is possible that BAFF/APRIL neutralization in our studies dampens CD40-independent help provided by memory CD4 T cells. Despite shared receptors and overlapping effects, BAFF and APRIL have unique functions during humoral immune responses. While BAFF signaling stabilizes germinal centers and prolongs B cell activation, APRIL is essential for the long term survival of plasma cells (23, 25, 30, 31). As GC B cell numbers in our study are not significantly affected at d. 14 posttransplant (Figure 2B), the decrease in alloantibody titers can be explained by the importance of BAFF and/or APRIL during germinal center reaction (as indicated by recent findings from Goenka et al., (32)) and plasma cell differentiation. Indeed, we found that mTACI-Fc decreased early spleen plasma cells (Figure 2B) that are the major source of anti-donor IgG alloAb early after transplantation (6) and had immediate effects on alloAb responses. In contrast, recipients treated with mBAFFR-Fc treatment demonstrated only modest declines in early alloAb responses but at the time of rejection had decreased alloAb titers comparable to those in mTACI-Fc treated recipients (Figure 2).

In addition to the effects on alloAb, BAFF or BAFF/APRIL neutralization inhibited donor-reactive T cells. Activated mouse and human T cells express BAFFR, but not TACI, and respond to BAFF costimulation by proliferation and IFN γ and IL-2 secretion (11, 13, 33). Nevertheless, in our studies mTACI-Fc treatment significantly decreased frequencies of donor-reactive IFN γ -producing T cells both early and late after transplantation, whereas maximal inhibition of T cell responses by mBAFFR-Fc was observed only at the time of rejection. These findings suggest that early defects in alloAb responses may lead to diminished tissue injury and less efficient antigen processing and presentation to recipient T cells. Another possibility is that antigen presentation by B cells is required for long term maintenance of both naïve and memory T cell responses. Using adoptive transfer of donor-reactive memory CD4 T cells of defined specificity allowed us to directly assess the effects of BAFF/APRIL neutralization on this subset. Notably, neutralization of BAFF or BAFF/APRIL not only inhibited activation of endogenous naïve T cells following transplantation but also interfered with the ability of established subsets of memory CD4 T cells to secrete IFN γ and differentiate into Tfh cells upon reactivation (Figures 4–5). This is a novel finding, as the T cell costimulatory properties of both BAFF and APRIL have never been examined during *in vivo* memory T cell reactivation.

The addition of either mBAFFR-Fc or mTACI-Fc to anti-CD154 mAb significantly prolonged heart allograft survival compared to anti-CD154 monotherapy in recipients containing donor-reactive memory CD4 T cells. As B cells have been recently established as important antigen presenting cells during primary alloimmune responses and during

differentiation of long-lasting T cell memory (34, 35), this prolongation could be attributed to the decline in B cell survival. However, we have previously demonstrated that the reactivation of donor-specific memory CD4 T cells and their pathogenic functions can occur independently of B cells. In particular, memory CD4 T cells induced rapid heart allograft rejection in B cell deficient recipients despite CD40/CD154 blockade (4). These results suggest that the observed effects of BAFF/APRIL neutralization in the current study are not entirely due to B cell depletion, but may also include direct influence on memory T cell functions. Consistent with the impact on both cellular and humoral immune responses, treatment with mTACI-Fc was more efficient in graft prolongation than mBAFFR-Fc administration, with nearly half of the recipients maintaining grafts beyond day 90. As mBAFFR-Fc or mTACI-Fc were administered only for two weeks after transplantation, these findings indicate that peri-transplant manipulation of memory CD4 T cell helper functions may have lasting effects and translate into marked improvement in transplant outcome.

After mBAFFR-Fc or TACI-Fc treatments were discontinued, the delayed immune responses were manifested by alloAb production. Both treatments shifted isotype distribution of donor-reactive alloAb from IgG2b and IgG2c to IgG1. While switching to IgG1 isotype is commonly associated with IL-4 signaling, we detected extremely low IL-4 production by HY-specific memory T cells in the treated recipients both at d. 14 and at rejection (Figure S2B and not shown). This suggests that the alloAb isotype shift may have resulted from the decreased IFN γ production by helper memory CD4 T cells (Figure 3). Importantly, the late increase in serum titers of donor-reactive alloAb was associated with prominent C4d deposition in the graft (Figure 4). Even though mouse IgG1 is commonly designated as a non-complement-activating isotype, low levels of IgG2b (as observed in mBAFFR-Fc and mTACI-Fc-treated recipients, Figure 2) synergize with IgG1 to activate complement on donor vascular endothelium (36). The role of induced alloAb during delayed rejection was further confirmed by the finding that recipient CD8 T cell depletion has little effect on the kinetics of allograft survival in this model (Figure 5).

The adoptive transfer approach used in our study is essential for understanding the mechanisms underlying the effects of BAFF and APRIL on memory CD4 T cell functions. However, sensitization through direct alloantigen exposure is often encountered in transplant patients and results in pre-existing donor-reactive memory CD4 and CD8 T cells as well as alloAb. Any of these mechanisms can trigger costimulation blockade-resistant transplant rejection. It is important to note that in clinical transplantation, fully sensitized patients are identified by a positive Ab cross match and are not likely candidates for costimulatory blockade treatment. Not surprisingly, BAFF/APRIL neutralization did not alter the high titers of circulating donor-reactive alloAb in skin-sensitized recipients and did not prevent antibody-mediated graft injury in our studies (Figure 8A, C). Nevertheless, it should be noted that treatment with mTACI-Fc plus anti-CD154 mAb significantly reduced *de novo* B cell responses under these conditions, analogous to our findings in an adoptive memory T cell transfer model.

In summary, therapeutic interference with the BAFF/APRIL network inhibits costimulatory blockade-resistant heart allograft rejection induced by donor-reactive memory CD4 T cells.

While BAFF and APRIL are commonly perceived as cytokines regulating humoral immunity with a possible role in T cell costimulation, this is the first report that neutralizing these factors inhibits CD40-independent reactivation and helper functions of donor-reactive memory CD4 T cells. Despite recent advances in immunosuppression, there is a large proportion of patients (up to 20%) that are PRA-negative and cross match-negative at the time of transplant, but later develop de novo pathogenic alloAb. Unfortunately there is no information available on the T cell sensitization status of such patients before transplantation. Several reagents blocking BAFF alone or BAFF in combination with APRIL are currently being tested in autoimmune disease patients (37). Our results suggest that these therapeutics may be used to enhance the efficacy of CD40/CD154 costimulatory blockade and improve allograft survival in T cell sensitized recipients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ab	antibody
ASC	antibody secreting cell
APRIL	a proliferation-inducing ligand
BAFF	B cell activating factor of the TNF family
MFI	mean fluorescence intensity
MST	mean survival time

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Figure 1. Treatment with mBAFFR-Fc or mTACI-Fc synergizes with anti-CD154 mAb to prolong heart allograft survival in CD4 T cell sensitized recipients

B6 mice containing Mar memory CD4 T cells were transplanted with BALB/c male heart allografts and treated with mBAFFR-Fc, TACI-Fc or control mFc4 either with (A) or without (B) anti-CD154 mAb MR1. N = 4–8 animals/group.

Figure 2. The effect of BAFF or BAFF/APRIL neutralization on B cell subsets
B6 mice containing Mar memory CD4 T cells were transplanted with BALB/c male heart allografts and treated with either anti-CD154 mAb MR1 alone or in combination with mBAFFR-Fc, TACI-Fc or control mFc4. Populations of B cells were analyzed in spleen on d. 14 posttransplant by flow cytometry. **A.** Gating strategy. **B.** Numbers of B220⁺CD21/35⁺IgM⁺ follicular B cells (FO), B220⁺CD21/35^{hi}IgM^{hi} marginal zone B cells (MZ), B220⁺CD21/35^{lo}IgM⁺ transitional B cells (TR), B220⁺CD38⁺ GL7⁺ germinal center B cells (GC) and B220⁻ CD138⁺ plasma cells (PC) in recipient spleen. N = 3 animals/group.

Figure 3. Neutralization of BAFF or BAFF/APRIL inhibits CD40-independent alloAb induction by memory CD4 T cells

B6 mice containing Mar memory CD4 T cells were transplanted with BALB/c male heart allografts and treated with either anti-CD154 mAb MR1 alone or in combination with mBAFFR-Fc, TACI-Fc or control mFc4. Serum titers of donor BALB/c or third party SJL-reactive IgG alloAb were determined d. 14 post-transplant, at the time of rejection or on d. 90 if rejection did not occur earlier. The titers of third party-reactive Ab were 45 for all analyzed IgG isotypes. N = 5–8 animals/group.

Figure 4. BAFF or BAFF/APRIL neutralization inhibits anti-donor T cell responses in recipients containing memory CD4 T cells

B6.CD45.2 mice containing CD45.1⁺Mar memory CD4 T cells were transplanted with BALB/c male heart allografts and treated with either anti-CD154 mAb MR1 alone or in combination with mBAFFR-Fc, TACI-Fc or control mFc4. B6 recipients of BALB/c heart allografts treated with MR1 mAb without Mar memory cell transfer served as an additional control. Recipients were sacrificed on d. 14 posttransplant, at the time of rejection or on d. 90 if rejection did not occur earlier. **A.** CD44, CD25 and CD69 expression at d. 14 posttransplant. Top: comparison of endogenous CD4 (CD45.2⁺) and transferred memory Mar (CD45.1⁺) cells in untreated recipients (no MR1, no BAFF/APRIL neutralization).

Bottom: the effect of MR1 with or without BAFF/APRIL neutralization on phenotype of transferred memory Mar cells. The results are representative of 3 animals/group. **B.** The frequencies of spleen cells producing IFN γ in response to donor BALB/c and third party SJL stimulator cells and to HY_{Dby} peptide were determined by ELISPOT assay. The numbers of cells producing IFN γ in response to the third party SLJ stimulators were $<50/1 \times 10^6$ spleen cells in all groups. N = 3–8 recipients/group.

Figure 5. Neutralizing BAFF/APRIL but not BAFF alone reduces Tfh cell differentiation
 B6.CD45.2 mice containing CD45.1⁺Mar memory CD4 T cells were transplanted with BALB/c male heart allografts and treated with either anti-CD154 mAb MR1 with or without mBAFFR-Fc or TACI-Fc. B6 recipients of BALB/c heart allografts treated with MR1 mAb without Mar memory cell transfer and naïve B6 mice served as an additional controls. **A.** Numbers of ICOS⁺PD-1⁺ follicular helper cells within transferred Mar and endogenous CD4 T cell populations were determined on d. 14 posttransplant. Detailed gating strategy is shown in Figure S3. **B.** The frequencies of spleen cells producing IL-21 in response to donor

BALB/c stimulator cells and to HY_{Dby} peptide were determined by ELISPOT assay. N = 3 animals/group.

Figure 6. Histological evaluation of heart allografts harvested at the time of rejection
H&E staining and immunohistochemical staining for CD3 and C4d were performed on paraffin-embedded tissue sections. The images are representative of 4–5 rejecting heart allografts analyzed in each group. Original magnification 200x.

Figure 7. CD8 T cell depletion does not alter the kinetics of heart allograft rejection in recipients treated with BAFF or BAFF/APRIL neutralizing reagents

B6 mice containing Mar memory CD4 T cells were injected with a cocktail of anti-mouse CD8 depleting mAb on d. -3, -2, -1 prior to transplantation and every 5 days thereafter throughout the duration of the experiment. CD8 T cell depleted recipients were transplanted with BALB/c male heart allografts and treated with either anti-CD154 mAb MR1 alone or in combination with mBAFFR-Fc, TACI-Fc or control mFc4. N = 6–9 recipients/group.

Figure 8. BAFF/APRIL neutralization inhibits *de novo* B cell responses but not heart allograft injury by preexisting alloAb

B6 mice were sensitized by placement of BALB/c skin allografts (all rejected by d. 14 posttransplant). Four weeks after skin transplantation, recipients were transplanted with BALB/c heart allografts and treated with anti-mouse CD8 depleting Ab (d. -3, -2, -1 and every 5 days thereafter) and with anti-CD154 mAb MR1 with or without mBAFFR-Fc or mTACI-Fc. **A.** Titers of serum donor-reactive IgG alloAb were evaluated in skin-sensitized B6 mice prior to heart transplantation (open circles) and at the time of heart allograft rejection (d. 5–7). The titers of third party SJL-reactive Ab were 45 for all analyzed IgG isotypes. **B.** The frequencies of total IgG or IgM ASCs and of ASCs producing alloAb against donor MHC class I (D^d) and class II (I-A^d) molecules in the spleen were determined by ELISPOT assay at the time of heart allograft rejection. **C.** Heart allografts were harvested at the time of rejection and C4d staining was performed on paraffin-embedded tissue sections. The images are representative of 5–7 rejecting heart allografts analyzed in each group. Original magnification 200x.